DOI: 10.1111/j.1439-0396.2008.00826.x

ORIGINAL ARTICLE

Fish protein substrates can substitute effectively for poultry by-product meal when incorporated in high-quality senior dog diets

K. E. Zinn[1], D. C. Hernot[1], N. D. Fastinger[1], L. K. Karr-Lilienthal[1], P. J. Bechtel[2], K. S. Swanson[1] and G. C. Fahey Jr[1]

1 Department of Animal Sciences, University of Illinois, Urbana, IL, USA, and
2 USDA, ARS, University of Alaska, Fairbanks, AK, USA

Keywords
dog, ageing, fish substrates, digestibility, immunity

Correspondence
G. C. Fahey Jr, Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA. Tel: (217) 333-2361; Fax: (217) 333-7861; E-mail gcfahey@uiuc.edu

Received: 16 November 2007; accepted: 5 March 2008

Summary

An experiment was conducted to analytically define several novel fish substrates and determine the effects of feeding diets containing these substrates on total tract nutrient digestibilities and on immune status of senior dogs. The control diet contained poultry by-product meal while test diets contained 20% milt meal (MM), pink salmon hydrolysate (PSH) and white fish meal (WFM) added at the expense of poultry by-product meal. Concentrations of lymphocytes positive for CD3, CD4, CD8α and CD21 cell-surface markers and immunoglobulin concentrations were measured. Gene expression of cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IL-10 and transforming growth factor (TGF)-β was determined by quantitative real-time polymerase chain reaction. Major compositional differences were noted among fish substrates but apparent nutrient digestibility coefficients and immune indices were not affected by treatment. Fish protein substrates were found to be effective substitutes for poultry by-product meal, providing diets of high nutritive value for senior dogs.

Introduction

The world pet food market offers a wide variety of commercial diets for companion animals. Diets are formulated to meet an animal's nutritional needs for different life stages, disease states and activities. Because of improved awareness of pet health and nutrition by owners, demand for high-quality foods has increased (Crossley, 2003).

Alternative protein sources have been sought to meet this demand for high-quality foods while ensuring the amino acid requirements of the animal. They also need to serve as palatants and (or) modulate immune function (Hayek and Davenport, 1998). A variety of animal by-products have been used as protein sources, but alternative sources such as fish by-products have also been used primarily in species other than companion animals.

Fish by-products are sources of high-quality protein and highly digestible essential amino and fatty acids. These characteristics allow their use in high-quality pet foods across all life stages. However, aged animals, with potentially compromised nutrient digestion and immune function, may benefit most from the use of fish by-products. Protein reserves are decreased in older animals, resulting in loss of lean body mass. These animals may require a constant supply of amino acids to meet anabolic requirements, thus increasing the need for high-quality protein in the diet (Sheffy and Williams, 1981). Nutritional intervention – including altering dietary concentrations and ratios of omega-6 (n-6) to omega-3 (n-3) fatty acids – may also improve the immune response of aged animals. The objectives of this study were to analytically define select fish substrates and to determine the digestibility and

immunomodulatory role of fish by-products included in senior dog diets.

Materials and methods

Fish substrates

The MM was obtained from Kodiak Fishmeal Company (Kodiak, AK, USA). Walleye pollock and pink salmon testes were collected, dried and ground. The PSH was prepared by Bio-Oregon (Warrenton, OR, USA). Fresh salmon by-products, including heads and viscera, were minced, cooked, deboned, and the aqueous fraction containing soluble protein and oil was separated from the precipitated protein. A commercial protease was added to the precipitated protein and hydrolysis was allowed for 60 min at 57° C. Hydrolysis was followed by heating to inactivate the proteolytic enzyme. Ethoxyquin and citric acid were added at 0.1% as antioxidants and the hydrolysate was dried to a powder.

The WFM was obtained from Kodiak Fishmeal Company and was prepared from the by-products of Alaska pollock with small amounts of Pacific cod and flat fish by-products. Fish by-products were ground and cooked for about 20 min at 90° C, then water was removed mechanically with a screw press, producing press cakes and press liquor. The press liquor was clarified through a decanter centrifuge and the supernatant (clarified liquor) was collected. The clarified liquor was then centrifuged to separate the oil and aqueous fraction. The aqueous fraction, with a solid content of 8–10% primarily composed of protein, was concentrated and added to the press cake. This material was then dried and milled.

Animals

Two female and 10 male senior pointers with a mean starting age of 94.6 (±14.9) months and a mean starting body weight of 24.0 (±2.4) kg were used. Dogs were housed at Kennelwood (Champaign, IL, USA). All dogs were allowed free access to water. The University of Illinois Institutional Animal Care and Use Committee approved the experimental protocol.

Diets

All dogs were fed commercial-type, high-protein diets that were prepared at Kansas State University Department of Grain Science and Industry (Manhattan, KS, USA) under the supervision of Pet Food & Ingredient Technology (Topeka, KS, USA). The diets were in extruded, dry kibble form. The control diet contained poultry by-product meal as the primary protein source. Test diets contained either 20% PSH, WFM or MM. Test ingredients were included at this concentration to avoid fish odour in the final product and to ensure that palatability was not negatively affected. The diets were formulated to contain 30% crude protein (CP) and 25% fat [dry matter (DM) basis] and to meet or exceed the National Research Council (2006) requirements for dogs and cats.

Experimental design

Three treatments were evaluated using a randomized complete block design in two 26-day blocks. All animals were fed the control diet from day 1 to 14, and then were allotted to one of the four treatment diets from day 15 to 26. Dogs were offered 500 g of diet daily on an as is basis and the amount offered each day was close to the calculated energy requirement of the dogs.

Sample collection

Total faeces excreted were collected on day 24, 25 and 26 from the pen floors. Faeces were weighed at the time of collection, frozen at −20° C, and composited by dog at the end of each block. Scoring was determined as follows: 1 = hard, dry pellets; 2 = dry, well-formed stool; 3 = soft, moist, formed stool; 4 = soft, unformed stool; 5 = watery, liquid that can be poured.

Blood samples collected on the last day of each period (day 14 and 26) via jugular venipuncture were analysed for concentrations of lymphocytes positive for CD3, CD4, CD8α and CD21 cell-surface markers; gene expression of interleukin (IL)-6, IL-10, interferon (IFN)-γ and transforming growth factor (TGF)-β cytokines; serum immunoglobulin concentrations for IgA, IgG and IgM; and complete blood count. Complete blood count was measured using a Cell-Dyn 3500 haematology analyser (Abbott Laboratories, Abbott Park, IL, USA). A sample of approximately 500 g of diet was taken from several bags of each of the diets, composited, and a 500-g subsample removed for analysis. Composited diet was stored at 4° C until analysis.

Chemical analyses

Faecal samples were dried in a forced air oven at 55° C. Diet and dried faecal samples were ground in

a Wiley mill (model 4; Thomas Scientific, Swedesboro, NJ, USA) through a 2-mm screen. Diet and faecal samples were analysed for DM and organic matter (OM) according to the methods of the Association of Official Analytical Chemists (AOAC) (1995). Fat concentrations were measured by acid hydrolysis [American Association of Cereal Chemists (AACC) (1983)]. Diet and fecal protein concentrations were measured according to AOAC using a LECO nitrogen analyser (model FP-2000; Leco Corporation, St. Joseph, MI, USA). Gross energy values of samples were determined using bomb calorimetry (Parr Instrument, Moline, IL, USA). Diet and substrate fatty acid compositions were measured according to Lepage and Roy (1986). Substrate amino acids were determined using an amino acid analyser (model 6300; Beckman Coulter, Fullerton, CA, USA) at the Experiment Station Laboratory, University of Missouri (Columbia, MO, USA).

Whole-blood staining method

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and prepared within 24 h for cell surface marker analysis. All blood-staining steps were carried out in the dark. Aliquots of 100 μl of uncoagulated blood were transferred to 12 × 75 mm tubes in duplicate for each CD marker. Each aliquot received 25 μl of monoclonal antibody and was vortexed before incubation for 15 min at room temperature.

Samples were kept on ice throughout the remainder of the analysis. Samples then were washed with 3 ml of FACS buffer (phosphate-buffered saline with 5 mM EDTA and 0.1% w/v sodium azide), centrifuged at 269 × *g* for 5 min at 4° C, and the supernatant discarded.

Three millilitres of lysing reagent (8.26 g NH_4Cl, 1.0 g $KHCO_3$, and 0.037 g Na_4EDTA in 1.0 l deionized-distilled H_2O; pH 7.2) was added to pelleted blood and vortexed to lyse red blood cells. Samples were incubated for 5 min at room temperature before being centrifuged (269 × *g*, 5 min, 4° C) and the supernatant removed. The lysing step was then repeated with 1.5 ml of lysing reagent.

Samples were then washed with 3 ml of FACS buffer, vortexed and re-centrifuged. After removing the supernatant, cells were resuspended in 10 μl of horse anti-mouse IgG-FITC (1:100 in FACS buffer; Vector Labs, Burlingame, CA, USA) and incubated in the dark for 20 min. Samples were then washed again with 3 ml of FACS buffer, re-centrifuged and the supernatant discarded.

The pelleted cells were then resuspended in 400 μl of FACS buffer by vortexing. Samples were fixed with 100 μl 2% w/v paraformaldehyde in PBS (pH 7.4) and stored at 4° C away from light until analysis using a Coulter Epics XL flow cytometer (Biotechnology Center, Flow Cytometry Facility, University of Illinois).

Gene expression determination

Blood samples were collected in PAXgene Blood RNA tubes (Qiagen, Valencia, CA, USA) and RNA was isolated according to the PAXgene Blood RNA Kit protocol. RNA was then converted to cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). PrimerExpress 2.0 software (Perkin-Elmer, Boston, MA, USA) was used to design primer-probe pairs specifically for the IFN-γ (forward primer, 5′-TCTCACCAAGATCCAACC-TAAGG-3′; reverse primer, 5′-TGCGGCCTCGAAA-CAGA-3′; probe, 5′-AGCGGAAAAGGAGTCAGA-3′), IL-6 (forward primer, 5′-GACCACTCCTGACC-CAACCA-3′; reverse primer, 5′-ATCCTGCGACTGCA-AGATAGC-3′; probe, 5′-ACGCCAGCCTGCA-3′), IL-10 (forward primer, 5′-CCCAGGATGGCAACTCT-TCTC-3′; reverse primer, 5′-CGGGATGGTATTTTGC-AGATC-3′; probe, 5′-CTCTAGGACATGAATTGG-3′), and TGF-β genes (forward primer, 5′-AGGTGGAGC-AGCTGTCGAA-3′; reverse primer, 5′-GGGCCTCA-GCTGCACTTG-3′; probe, 5′-ATGATCGTGCGCTCC-3′). Quantitative real-time polymerase chain reaction (PCR) was performed using a Custom TaqMan® Gene Expression PCR Kit (Qiagen) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) housed in the Functional Genomics Laboratory, W.M. Keck Center for Comparative and Functional Genomics, University of Illinois. The genes were tested in triplicate and a standard curve was plotted using a control sample as a calibrator. Primer-probe pairs for 18S ribosomal RNA were amplified in parallel with IFN-γ, IL-6, IL-10 and TGF-β as a control for all samples. Unknown gene threshold cycle (Ct) values were corrected by subtracting the corresponding 18S gene to determine a ΔCt value. ΔCt values were then subtracted from baseline ΔCt to determine $\Delta\Delta$Ct values. Fold change from baseline was determined from the $\Delta\Delta$Ct values.

Serum immunoglobulin concentration

Blood samples were collected into serum separator evacuated tubes and centrifuged at 2000 × *g* at 4° C for 20 min and the serum stored at −80° C until

analysis. Samples were analysed for IgA, IgG and IgM according to the VET-RID Kit protocol (Bethyl Laboratories, Montgomery, TX, USA).

Calculations

Apparent total tract nutrient digestibilities were calculated as: nutrient intake (DM basis) − nutrient output (DM basis)/nutrient intake (DM basis) × 100%.

Statistical analysis

Data were analysed as a randomized complete block design using the mixed models procedure of SAS (SAS Inst., Cary, NC, USA). The statistical model included the fixed effect of dietary treatment and the random effects of both block and animal. Treatment least squares mean values are reported and were compared using LSD adjustment. Differences among mean values with a *P*-value of less than 0.05 were considered statistically significant.

Results

Diets and substrates

Chemical composition of the fish protein substrates is presented in Table 1. Percentage DM ranged from 82.9% to 92.8%, with MM having the lowest and WFM having the highest DM. Percentage OM ranged from 78.4% to 94.0%, with WFM having the lowest and PSH having the highest OM. The hydrolysate contained less ash than meals. Crude protein concentrations of fish substrates ranged from 70% for WFM to 85.3% for MM.

Acid-hydrolysed fat values ranged from 12.1% to 20.3%, with WFM having the lowest and PSH having the highest. Crude fat values are generally lower than acid-hydrolysed fat values because they do not measure bound lipids like cell-membrane phospholipids. Gross energy values ranged from 4.6 (WFM) to 5.9 kcal/g (PSH).

White fish meal contained the greatest concentration of total biogenic amines (2716.8 μg/g) and MM contained the lowest concentration (80.8 μg/g). Total amino acid concentrations varied widely among substrates, as did total essential and non-essential amino acid concentrations. Total amino acid concentrations ranged from 39.5% (MM) to 53.8% (WFM).

Fatty acid composition of the substrates is presented in Table 2. Fatty acid composition varied among substrates, with the highest concentrations of fatty acids in PSH (128.1 mg/g) and the lowest in WFM (88.8 mg/g). The n-6 to n-3 ratio was the same among substrates, with a value of 0.1. Milt meal and PSH had similar concentrations of EPA and

Table 1 Proximate constituents, amino acid and biogenic amine concentrations, and gross energy content of fish protein substrates fed to senior dogs

Item	Milt meal	Pink salmon hydrolysate	White fish meal
Dry matter, %	82.9	87.5	92.8
		%, dry matter basis	
Organic matter	88.0	94.0	78.4
Crude protein	85.3	78.7	70.0
Crude fat	12.1	16.2	7.7
Acid hydrolysed fat	15.1	20.3	12.1
Gross energy, kcal/g	5.6	5.9	4.6
Biogenic amines, μg/g			
Agamatine	0.0	8.1	0.0
Cadaverine	0.4	226.3	1066.0
Histamine	0.0	3.0	116.7
Phenylethylamine	0.0	0.0	70.1
Putrescine	80.4	224.1	502.5
Spermidine	0.0	104.5	0.0
Spermine	0.0	11.0	0.0
Tyramine	0.0	190.3	961.5
Total biogenic amines	80.8	767.3	2716.8
Essential amino acids			
Arginine	3.6	4.5	3.7
Histidine	0.9	1.3	1.1
Isoleucine	1.6	2.6	2.1
Leucine	3.3	4.5	3.8
Lysine	4.0	4.6	3.8
Methionine	0.9	1.6	1.5
Phenylalanine	1.5	2.4	2.0
Threonine	2.0	2.4	2.3
Tryptophan	0.5	0.9	0.6
Valine	2.3	3.1	2.5
Non-essential amino acids			
Alanine	2.6	3.2	3.6
Aspartic acid	2.9	4.9	4.8
Cysteine	0.4	0.6	0.5
Glutamic acid	4.7	6.3	7.0
Glycine	2.4	3.0	5.5
Hydroxylysine	0.0	0.1	0.3
Hydroxyproline	0.0	0.2	1.1
Lanthionine	0.0	0.1	0.0
Ornithine	0.0	0.1	0.1
Proline	1.7	2.2	3.0
Serine	1.6	2.1	2.5
Taurine	1.2	0.3	0.4
Tyrosine	1.3	2.1	1.7
TEAA	20.7	27.9	23.4
TNEAA	18.8	25.2	30.5
TAA	39.5	53.2	53.8

TEAA, total essential amino acids; TNEAA, total non-essential amino acids; TAA, total amino acids.

Table 2 Fatty acid concentrations of fish protein substrates fed to senior dogs

Fatty acids, mg/g	Milt meal	Pink salmon hydrolysate	White fish meal
DHA	15.5	15.6	7.5
EPA	11.1	11.2	5.9
Total SFA	29.4	38.7	25.4
Total MUFA	30.9	46.2	41.3
Total n-3 PUFA	36.6	36.0	17.7
Total n-6 PUFA	2.5	4.5	2.3
Total PUFA	39.4	42.3	21.5
Total iso-BCFA	0.1	0.7	0.5
Total anteiso-BCFA	0.0	0.3	0.2
Total BCFA	0.1	1.0	0.7
Total fatty acids	99.8	128.1	88.8
n-6:n-3	0.1	0.1	0.1

DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; BCFA, branched-chain fatty acids.

DHA (15.5 and 11.1 mg/g, 15.6 and 11.2 mg/g respectively) while WFM had the lowest concentrations (7.5 and 5.9 mg/g respectively).

Ingredient and chemical compositions of diets are presented in Table 3. Diets differed in DM and OM concentration by approximately three percentage units each. Higher ash concentration occurred in the control and WFM diets, largely because of a greater amount of bone present in the major dietary protein sources. Percentage CP ranged from 29.8% in the MM diet to 30.9% in the control diet. Acid-hydrolysed fat concentration varied from 23.8% in the PSH diet to 27.2% in the MM diet.

Intake, digestibility and faecal characteristics

Average daily food intakes, apparent nutrient digestibility values, and faecal characteristics are presented in Table 4. Average daily food intakes were similar among treatments throughout the study, with the dogs ingesting nearly all the food they were provided. Apparent nutrient digestibility coefficients were high and did not differ among treatments. There were no differences in faecal output expressed on an as is or DM basis, or when corrected for differences in DM intake. The faecal DM concentration was higher ($p < 0.05$) for dogs fed the control and WFM diets than for those fed the remaining treatments, perhaps because of the higher ash contents in the two diets. Faecal scores were the highest ($p < 0.05$) for dogs fed MM and the lowest for dogs fed WFM. Other values were intermediate.

Table 3 Ingredient and chemical compositions of diets fed to senior dogs

	Diet			
Item	Control	Milt meal	Pink salmon hydrolysate	White fish meal
Ingredient composition				
Brewer's rice	43.69	44.45	47.56	43.81
Poultry by-product meal	35.06	10.40	11.93	13.41
Poultry fat	13.98	17.88	13.24	15.51
Fish protein substrate	–	20.0	20.0	20.0
Beet pulp	4.0	4.0	4.0	4.0
Dried egg	2.0	2.0	2.0	2.0
Potassium chloride	0.5	0.5	0.5	0.5
Salt	0.4	0.4	0.4	0.4
Choline chloride	0.13	0.13	0.13	0.13
Vitamin mix*	0.12	0.12	0.12	0.12
Mineral mix†	0.12	0.12	0.12	0.12
Chemical composition				
Dry matter, %	92.1	94.5	94.2	94.5
		%, dry matter basis		
Organic matter	92.9	94.7	94.7	92.3
Crude protein	30.9	29.8	30.6	30.4
Acid-hydrolysed fat	24.3	27.2	23.8	24.7
Gross energy, kcal/g DM	5.4	5.6	5.4	5.3

*Provided per kilogram of diet (vitamin form): vitamin A (vitamin A acetate), 12 470 IU; vitamin D (vitamin D_3), 750 IU; vitamin E (vitamin E supplement), 49.9 IU; vitamin K (menadione sodium bisulfite complex), 0.5 mg; thiamin (thiamine mononitrate), 10.0 mg; riboflavin (riboflavin supplement), 8.0 mg; pantothenic acid (d-calcium pantothenate), 15.0 mg; niacin (niacin supplement), 37.4 mg; vitamin B_6 (pyridoxine hydrochloride), 10 mg; biotin (biotin supplement), 0.1 mg; folic acid (folic acid supplement), 0.6 mg; vitamin B_{12} (vitamin B_{12} supplement), 0.01 mg; choline (choline chlorine), 199.6 mg.

†Provided per kilogram of diet (mineral form): manganese (manganese sulphate), 3.0 mg; iron (ferrous sulphate), 22.5 mg; copper (copper sulphate), 3.0 mg; cobalt (cobalt sulphate), 0.6 mg; zinc (zinc sulphate), 30.0 mg; iodine (potassium iodide), 0.38 mg; selenium (sodium selenite), 0.06 mg.

Immune function measurements

Complete blood chemistry profile data are not shown. Monocyte concentration change from baseline was higher ($p < 0.05$) for dogs fed the control and WFM diets. Values were within published reference ranges listed in the Merck Veterinary Manual (Merck Handbooks, 1998). Lymphocyte subset concentration data are presented in Table 5. No differences were found among treatments for any of the cell-surface markers analysed. All values were within published ranges (Faldyna et al., 2001, 2005). Fold change from baseline to treatment in cytokine gene expression data are shown in Table 6. No significant differences were found among treatments as regards

Table 4 Average daily food intakes, apparent total tract nutrient digestibility coefficients, and fecal characteristics of senior dogs fed diets containing fish protein substrates*

Item	Diet				SEM†
	Control	Milt meal	Pink salmon hydrolysate	White fish meal	
Intake, g/day					
Animal, no./treatment	5	6	6	5	
Baseline	477	499	499	471	8.8
Treatment	491	497	495	499	4.7
Apparent digestibility, %					
Dry matter	88.6	90.9	90.9	88.2	1.3
Organic matter	91.6	92.6	93.0	92.0	1.0
Crude protein	86.0	88.6	88.4	88.0	1.6
Acid-hydrolysed fat	96.5	97.0	96.8	96.7	0.4
Gross energy	93.1	93.8	93.9	93.2	0.8
Faecal characteristics					
Faecal output (as is), g/day	190.0	204.3	179.1	203.4	26.4
Faecal output (DM), g/day	51.1	42.8	41.5	54.8	5.9
Faecal output (as is) per g DM consumed	0.42	0.43	0.39	0.43	0.06
Faecal DM, %	28.6 b	22.6 a	24.3 a	28.2 b	1.2
Faecal score‡	3.5 ab	3.7 b	3.3 a	3.1 a	0.2

*Values are least squares means. Mean values in the same row with different letters differ ($p < 0.05$).
†Standard error of the mean.
‡Scores based on the following scale: 1 = hard, dry pellets; 2 = dry, well-formed stool; 3 = soft, moist, formed stool; 4 = soft, unformed stool; 5 = watery, liquid that can be poured.

Table 5 Lymphocyte subset concentrations for senior dogs fed diets containing fish protein substrates*

Item	Reference range‡	Diet				SEM†
		Control	Milt meal	Pink salmon hydrolysate	White fish meal	
Animal, no./treatment		5	6	6	5–6	
CD3, %	78.3–88.9	82.5	82.5	82.7	83.1	1.7
CD4, %	36.1–44.5	37.1	36.6	35.7	38.1	2.9
CD8, %	10.7–26.9	18.3	19.0	17.1	16.1	2.2
CD21, %	8.7–21.3	9.9	13.5	11.6	12.1	1.9
CD4:CD8	0.1–5.2	2.2	2.7	2.6	3.9	1.0

*Values are least squares means.
†Standard error of the mean.
‡Reference ranges according to Faldyna et al. (2001, 2005).

Table 6 Fold change from baseline in cytokine gene expression from day 14 to day 26 for senior dogs fed diets containing fish protein substrates*

Item	Diet				SEM†
	Control	Milt meal	Pink salmon hydrolysate	White fish meal	
Fold change from baseline					
Animal, no./treatment	5	6	6	4	
IFN-γ	1.1	1.5	1.2	1.1	0.3
IL6	1.1	1.5	1.4	1.5	0.5
IL10	0.9	1.5	1.2	1.2	0.3
TGF-β	0.7	1.0	1.1	0.8	0.1

*Values are least squares means.
†Standard error of the mean.

IL-6, IL-10, TGF-β or IFN-γ levels. Serum immunoglobulin concentration data are shown in Table 7. During the treatment period, we found no statistical differences among treatments for any of the immunoglobulins despite higher IgA concentrations for dogs fed the MM and WFM diets compared with those fed the control and PSH diets. There was also a significantly higher ($p < 0.05$) IgA concentration change from baseline for the MM and PSH diets compared with the WFM diet.

Discussion

We found some differences in the chemical composition of the fish protein substrates tested in this study. Total amino acid concentrations varied widely among substrates (from 39.5% to 53.8% for MM and WFM respectively), as did total essential and non-essential amino acid concentrations. This variation may be due to the fish species, as well as the processing methods used to prepare the hydrolysates and meals. Total amino acid concentrations were lower than CP values for all protein substrates, in

Table 7 Serum immunoglobulin concentrations of senior dogs fed diets containing fish protein substrates*

Item	Diet				
	Control	Milt meal	Pink salmon hydrolysate	White fish meal	SEM†
Animal, no./treatment	5	6	6	6	
Baseline					
IgA, mg/dl	215.7 a	234.3 a	213.4 a	272.0 b	41.9
IgG, mg/dl	2454.4	2418.2	2297.0	2496.9	240.1
IgM, mg/dl	288.5	315.3	318.9	308.5	35.6
Treatment					
IgA, mg/dl	220.4	258.3	224.1	251.3	35.1
IgG, mg/dl	2303.3	2388.3	2191.1	2309.8	233.0
IgM, mg/dl	330.1	302.3	319.0	310.9	28.7
Change					
IgA, mg/dl	0.3 ab	23.8 b	9.0 b	−17.7 a	7.6
IgG, mg/dl	−155.8	5.1	−135.6	−184.7	119.2
IgM, mg/dl	33.3	−11.8	8.8	2.4	20.3

*Values are least squares means. Mean values in the same row with different letters differ ($p < 0.05$).

†Standard error of the mean.

agreement with the data of Folador et al. (2006). This difference is due to substrates containing non-protein-nitrogen compounds such as biogenic amines, purines, pyrimidines and nucleotides.

Biogenic amines are the products of microbial decarboxylation of amino acids such as Arg, His, Lys, Trp and Tyr. White fish meal contained the highest concentration of amines (2716.8 μg/g) and MM contained the lowest concentration (80.8 μg/g). The substrates with higher biogenic amine concentrations would have the greatest microbial decarboxylation of amino acids. Putrescine, histamine, cadaverine and tyramine concentrations are indicators of raw fish freshness and serve as quality indicators for fish meal (Opstvedt et al., 2000). Studies do not define toxic levels of biogenic amines for companion animals. Despite important differences in total biogenic amine contents among the fish substrates, we did not find any differences in chemical composition of the diets among treatments. Moreover, according to our results, the range of biogenic amine concentrations in the fish products did not seem to alter nutrient digestibility or immune status of our dogs.

The n-3 PUFAs are of interest primarily because of their alleged health benefits. These fatty acids are found in aquatic resources (algae, fish and marine mammals) almost exclusively, in varying amounts and ratios (Shahidi, 2003). Gruger et al. (1964) showed wide variations in fatty acid composition among fish species, also indicating differences caused by climate and season. Eicosapentaenoic acid (EPA) concentration ranged from 5.0% to 21.5% among the species analysed, including pink salmon, atlantic cod, lake whitefish and menhaden. Docosahexaenoic acid (DHA) values ranged from 5.9% to 26.2%. The production of EPA and DHA via linolenic acid chain elongation is nearly nonexistent in adult dogs (Bauer et al., 2004). Providing long-chain n-3 PUFAs (EPA and DHA) is more effective than adding linolenic acid to the diet of senior dogs. Milt meal and PSH had similar concentrations of EPA and DHA while WFM had the lowest concentrations. These differences are to be expected as WFM would have low concentrations because of the removal of oil during processing.

In our study, diets were of high quality and formulated to be isonitrogenous and isocaloric. No differences in chemical compositions were found among diets. Percentage CP ranged from 29.8% (MM diet) to 30.9% (control diet) and gross energy values differed by only 0.3 kcal/g.

Average daily food intakes were similar among treatments throughout the study, with the dogs ingesting nearly all the food they were provided. This indicates that the concentrations of fish by-products included did not negatively affect palatability of the diets. There was a mild to moderate fish odour associated with the test diets which did not appear to have an impact on food intake. Apparent nutrient digestibility coefficients were high and not different among treatments, indicating the absence of anti-nutritive compounds in all fish substrates tested at the 20% dietary inclusion level. The high non-protein nitrogen component in MM may lead to production of more moist feces whereas the relatively low concentration

in WFM may result in drier excreta. Interestingly, dogs fed both fish substrates produced nearly identical quantities of wet stool.

As there were only differences in monocyte concentration change from baseline for dogs fed the control and WFM diets, and no significant differences during the treatment period, the inclusion of fish substrates to dog diets appears to have had little effect on the blood components measured in the complete blood count. Studies reviewed by Calder (1998) in humans and animals have shown that consumption of n-3 PUFAs, especially from fish oils, decreases lymphocyte proliferation, T-cell-mediated cytotoxicity, antigen presentation, and production of pro-inflammatory cytokines. Hall et al. (2003) showed higher ($p < 0.05$) lymphocyte counts in dogs (age 7–10 years) fed diets with a low n-6:n-3 ratio (1.4:1) compared with dogs fed diets with a high n-6:n-3 ratio (40:1) for 17 weeks. There were no significant differences in percentages of $CD4^+$ or $CD8^+$ T cells for dogs fed high n-6:n-3 diets compared with those in dogs fed low n-6:n-3 diets. Lymphocyte proliferation and PGE_2 production were lower ($p < 0.05$) in rats fed diets containing either 4.4 g EPA + DHA or 6.6 g EPA + DHA compared with rats fed a control diet containing 4.4 g α-linolenic acid, all containing an approximately 7:1 n-6 to n-3 ratio (Peterson et al., 1998). These studies showed some different effects on lymphocyte counts, but used different species and different n-6 to n-3 dietary ratios. Other studies observed lymphocyte population changes after a longer treatment period (Hall et al., 1999, 2003; Kearns et al., 1999), which may explain the lack of differences because of treatment in this study. Our results indicate no effect of dietary treatments on percentages of B and T cells. The percentages of cells displaying the different CD markers were within published ranges, indicating that the dogs were healthy during the study and had similar cell percentages previously noted in senior dogs (Faldyna et al., 2001, 2005).

Kearns et al. (1999) found no differences in IL-6 bioactivity in either young (mean age, 1.5 years) or old (mean age, 9.6 years) Laborador Retrievers, or young (mean age, 1.8 years) or old (mean age, 11.5 years) Fox Terriers when fed diets containing an n-6 to n-3 ratio of 5:1 for 8 weeks. Reports of the effect of n-3 PUFA-containing oils on rodent cytokine production range from no effect to enhanced production for a variety of cytokines. Fernandes et al. (1994) found elevated concentrations of mRNA for IL-2, IL-4 and TGF-β, which are anti-inflammatory, in the spleen of autoimmune disease-prone mice fed diets containing fish oil. Chandrasekar and Fernandes (1994) reported that dietary fish oil abolished mRNA production for IL-1β, IL-6 and TNF-α, which are pro-inflammatory, in mice. These effects would be expected to be applicable to all anti- and pro-inflammatory cytokines, though possibly to varying degrees. Published data regarding the effect of n-3 PUFAs on cytokine gene expression indicate a mechanism for increasing anti-inflammatory cytokine production. While these effects were not noted in this study, duration of the treatment period may not have allowed for detection of altered cytokine gene expression. Tappia and Grimble (1994) found reduced production of IL-1 and enhanced production of IL-6, both pro-inflammatory cytokines, when rats received fish oil for 4 weeks. Endres et al. (1989) found that supplementing the diet of human volunteers with 18 g of a fish oil concentrate for 6 weeks reduced the ability of monocytes to produce IL-1α and -β, and TNF-α, both pro-inflammatory cytokines.

We found no significant differences among treatments for IgG and IgM but a higher ($p < 0.05$) IgA concentration at baseline for the WFM treatment compared with the control, PSH, and MM diets. While no study has looked specifically at immunoglobulin concentrations after fish by-product or n-3 fatty acid inclusion in the diet, Greeley et al. (2001) reported that the total numbers of lymphocytes declined ($p < 0.01$) in dogs (age 4–11 years) from 7 to 11 years of age, and the percentage of B cells declined ($p < 0.05$) over an 8-year time period. This decrease in B cells would decrease the binding of immunoglobulins and decrease antibody production. Decreased binding of immunoglobulins could potentially lead to increased immunoglobulin concentrations in the peripheral blood.

The results of this experiment suggest that inclusion of fish by-products at the 20% inclusion level when substituted for poultry by-product meal in the diets of healthy, senior dogs does not dramatically impact nutrient digestibility or immune function. The lack of an effect on immune function may have resulted because the length of the treatment period was not sufficient to elicit a response in the variables measured. It is also possible that diets including fish by-products may more effectively alter immune function in diseased or challenged animals.

References

American Association of Cereal Chemists (AACC), 1983: *Approved Methods*, 8th edn. AACC, St. Paul, MN.

Association of Official Analytical Chemists (AOAC), 1995: *Official Methods of Analysis*, 15th edn. AOAC, Arlington, VA.

Bauer, J. E.; Heinemann, K. M.; Bigley, K. E.; Lees, G. E.; Waldron, M. K., 2004: Maternal diet α-linolenic acid during gestation and lactation does not increase docosahexaenoic acid in canine milk. *Journal of Nutrition* **134**, 2035S–2038S.

Calder, P. C., 1998: Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. *Brazilian Journal of Medical and Biological Research* **31**, 467–490.

Chandrasekar, B.; Fernandes, G., 1994: Decreased pro-inflammatory cytokines and increased antioxidant enzyme gene expression by ω-3 lipids in murine lupus nephritis. *Biochemical and Biophysical Research Communications* **200**, 893–898.

Crossley, A., 2003: The petfood market: noteworthy trends. *Petfood Industry* **45**, 14–16.

Endres, S.; Ghorbani, R.; Kelly, V. E.; Georgilis, K.; Lonnemann, G.; Van der Meer, J. W. M.; Rogers, J. G.; Klempner, T. S.; Weber, P. C., 1989: The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of IL-1 and TNF-α by mononuclear cells. *New England Journal of Medicine* **320**, 266–271.

Faldyna, M.; Leva, L.; Knotigova, P.; Toman, M., 2001: Lymphocyte subsets in peripheral blood of dogs – a flow cytometric study. *Veterinary Immunology and Immunopathology* **82**, 23–37.

Faldyna, M.; Sinkora, J.; Knotigova, P.; Leva, L.; Toman, M., 2005: Lymphatic organ development in dogs: major lymphocyte subsets and activity. *Veterinary Immunology and Immunopathology* **104**, 239–247.

Fernandes, G.; Bysani, C.; Venkatraman, J. T.; Tomar, V.; Zhao, W., 1994: Increased TGF-β and decreased oncogene expression by ω-3 fatty acids in the spleen delays onset of autoimmune disease in B/W mice. *Journal of Immunology* **152**, 5979–5987.

Folador, J. F.; Karr-Lilienthal, L. K.; Parsons, C. M.; Bauer, L. L.; Utterback, P. L.; Schasteen, C. S.; Bechtel, P. J.; Fahey, G. C. Jr, 2006: Fish meals, fish components, and fish protein hydrolysates as potential ingredients in pet foods. *Journal of Animal Science* **84**, 2752–2765.

Greeley, E. H.; Ballam, J. M.; Harrison, J. M.; Kealy, R. D.; Lawler, D. F.; Segre, M., 2001: The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. *Veterinary Immunology and Immunopathology* **82**, 57–71.

Gruger, E. H. Jr; Nelson, R. W.; Stansby, M. E., 1964: Fatty acid composition of oils from 21 species of marine fish, freshwater fish and shellfish. *Journal of the American Oil Chemists' Society* **41**, 662–667.

Hall, J. A.; Wander, R. C.; Gradin, J. L.; Du, S. H.; Jewell, D. E., 1999: Effect of dietary n-6 to n-3 fatty acid ratio on complete blood and total white blood cell counts, and T-cell subpopulations in aged dogs. *American Journal of Veterinary Research* **60**, 319–327.

Hall, J. A.; Tooley, K. A.; Gradin, J. L.; Jewell, D. E.; Wander, R. C., 2003: Effects of dietary n-6 and n-3 fatty acids and vitamin E on the immune response of healthy geriatric dogs. *American Journal of Veterinary Research* **64**, 762–772.

Hayek, M. G.; Davenport, G. M., 1998: Nutrition and aging in companion animals. *Journal of Anti-Aging Medicine* **1**, 117–123.

Kearns, R. J.; Hayek, M. G.; Turek, J. J.; Meydani, M.; Burr, J. R.; Greene, R. J.; Marshall, C. A.; Adams, S. A.; Borgert, R. C.; Reinhart, G. A., 1999: Effect of age, breed and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. *Veterinary Immunology and Immunopathology* **69**, 165–183.

Lepage, G.; Roy, C. C., 1986: Direct transesterification of all classes of lipids in a one-step reaction. *Journal of Lipid Research* **27**, 114–120.

Merck Handbooks, 1998: In: S. Aiello (ed.), *The Merck Veterinary Manual*, 8th edn. Merck, Whitehouse Station, NJ.

National Research Council, 2006: *Nutrient Requirements of Dogs and Cats*. The National Academies Press, Washington, DC.

Opstvedt, J.; Mundheim, H.; Nygard, E.; Aase, H.; Pike, I. H., 2000: Reduced growth and feed consumption of Atlantic salmon (*Salmo salar* L.) fed fish meal made from stale fish is not due to increased content of biogenic amines. *Aquaculture* **188**, 323–337.

Peterson, L. D.; Jeffery, N. M.; Thies, F.; Sanderson, P.; Newsholme, E. A.; Calder, P. C., 1998: Eicosapentaenoic and docosapentaenoic acids alter rat spleen leukocyte fatty acid composition and prostaglandin E2 production but have different effects on lymphocyte functions and cell-mediated immunity. *Lipids* **33**, 171–180.

Shahidi, F., 2003: Nutraceuticals and bioactives from seafood byproducts. In: P. J. Bechtel (ed), *Advances in Seafood Byproducts: 2002 Conference Proceedings*. Alaska Sea Grant College Program, Univ. Alaska, Fairbanks, AK, pp. 247–263.

Sheffy, B. E.; Williams, A. J., 1981: Nutrition and the aging animal. *The Veterinary Clinics of North America: Small Animal Practice* **11**, 669–675.

Tappia, P. S.; Grimble, R. F., 1994: Complex modulation of cytokine induction by endotoxin and tumor necrosis factor from peritoneal macrophages of rats by diets containing fats of different saturated, monounsaturated and polyunsaturated fatty acid composition. *Clinical Science* **87**, 173–178.